AD ______________

Award Number: DAMD17-01-1-0432

TITLE: Phosphatidylinositol 3-Kinane and Protein Kinase C as Molecular Determinants of Chemoresistance in Breast Cancer

PRINCIPAL INVESTIGATOR: Amanda P. Parker
Barbara Beckman, Ph.D.

CONTRACTING ORGANIZATION: Tulane University Health Sciences Center
New Orleans, LA 70112

REPORT DATE: July 2003

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2003	Annual Summary(1 Jul 2002 - 30 Jun 2003)

4. TITLE AND SUBTITLE

Phosphatidylinositol 3-Kinane and Protein Kinase C as Molecular Determinants of Chemoresistance in Breast Cancer

5. FUNDING NUMBERS

DAMD17-01-1-0432

6. AUTHOR(S)

Amanda P. Parker
Barbara Beckman, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Tulane University Health Sciences Center
New Orleans, LA 70112

E-Mail: mandaparker@hotmail.com

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT	12b. DISTRIBUTION CODE
Approved for Public Release; Distribution Unlimited	

13. ABSTRACT (Maximum 200 Words)

The goal of this study is to investigate the role of PI3K and PKC in chemoresistance in breast cancer and to use this information to identify novel therapeutic strategies aimed at counteracting or reversing drug resistance in breast cancer. We are using an isogenic model of breast cancer resistance with apoptosis-sensitive and apoptosis-resistant MCF-7 cells. Our preliminary data implicate the absence of PKCδ, and a constitutive activation of the PKCα and PI3K-Akt/NF-κB pathways as potential survival signals in our recently derived TNF-resistant MCF-7TN-R cells. Pharmacological inhibition of PKC and PI3K led to a decrease in cell viability in both chemoresistant and chemosensitive cell variants, but did not alter cellular sensitivity to TNF-α. However, we determined that the sphingolipid ceramide, a natural inhibitor of PKC and PI3K, was able to induce cell death equally in both cells variants, suggesting that it is a tool to overcome chemoresistance in these cells. Cell death occurred through activation of the mitochondrial pathway of apoptosis. Treatment with ceramide resulted in a decreased the phosphorylation of Akt, but did not alter the phosphorylation of PKC. Last, the efficacy of ceramide to induce apoptosis was increased using novel ceramide analogs.

14. SUBJECT TERMS	15. NUMBER OF PAGES
apoptosis and cell Survival, Protein Kinase C, chemotherapeutic, cellular signaling	11
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

ANNUAL REPORT

Amanda P. Parker (aparker2@tulane.edu)
Barbara S. Beckman (bbeckman@tulane.edu)
Department of Pharmacology
Tulane University Health Science Center
1430 Tulane Ave, SL-83
New Orleans, LA 70112-2699

Award Number: DAMD17-01-1-0432

INTRODUCTION

Chemotherapeutic drug resistance, or failure to initiate apoptosis as a response to a chemotherapeutic drug, results, in part, from a shift in the regulation of cellular mechanisms away from apoptosis to a more survival-oriented pathway. A diminished susceptibility to apoptosis may be mediated by the differential expression of certain key proteins, which serve as molecular determinants for the cancer cells' capacity to survive environmental stress, including drug treatment. Two proteins that have been implicated as anti-apoptotic are protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K). Although differential expression of these kinases have been linked to anti-apoptosis signaling mechanisms, the molecular details of the upstream and downstream events are not well-understood, and therefore elucidation of their mechanism of action may represent a potential therapeutic target for breast cancer. Using an isogenic model system of estrogen receptor positive, apoptosis-sensitive and apoptosis-resistant breast cancer cell variant MCF-7 cells, this proposal aims to define the role of PI3K and specific PKC isoforms in cellular apoptotic signaling pathways. It is expected that a detailed understanding of the role of these two kinases in breast cancer apoptotic signaling will provide opportunities for novel pharmacological interventions in *in vivo* systems.

STATEMENT OF WORK

The goal of this study is to investigate the role of PI3K and PKC in chemoresistance in breast cancer and to use this information to identify novel therapeutic strategies aimed at counteracting or reversing drug resistance in breast cancer. We are using an isogenic model of breast cancer resistance with apoptosis-sensitive and apoptosis-resistant MCF-7 cells, with resistance to apoptosis measured by resistance to TNF-α. Our preliminary data strongly implicate the constitutive expression/activity of PKCα, the absence of PKCδ, and a constitutive activation of the PI3K-Akt/NF-κB pathway as potential survival

signals in our recently derived TNF-resistant MCF-7TN-R cells. As outlined in Task 1, we investigated the relationship of PI3K and PKC and cell survival using pharmacological inhibitors of these kinases. Since our data indicate that PKC α is over-expressed in our resistant cell variant, we used two commercially available inhibitors of the conventional PKC isoforms, Gö6976 and Ro-32-0432, to determine the effect of PKC inhibition on MCF-7 variant viability and chemoresistance. Treatment of MCF-7 cell variants with the PKC inhibitors decreased MCF-7 cell number in both chemoresistant and chemosensitive MCF-7 cells (Appendix, Fig.1A), however inhibition of PKC did not restore sensitivity of the resistant cell variant to TNF-α (Appendix, Fig 1B). The results of treatment with MCF-7 cell variants with the PI3K inhibitor LY294002 were similar. LY294002 caused a dose-dependent decrease in MCF-7 cell number in both cell variants, while the sensitivity to TNF-α remained low in the apoptosis-resistant MCF-7TN-R variant (Appendix, Fig. 2A). These data demonstrate that both PKC and PI3K play an important role in MCF-7 cell proliferation and survival. However, from this data is does not appear that inhibition of either PKC or PI3K with the above inhibitors can lead to a reversal of MCF-7 chemoresistance.

Although we determined that PKC and PI3K were important in cell survival, neither inhibition of PKC nor PI3K alone had the desired effect of reversing chemoresistance in our MCF-7TN-R cells. While characterizing the TNF-resistant MCF-7TN-R cell line, we found that there was a marked decrease in ceramide (Cer) production in these cells following treatment with TNF-α, as compared to the sensitive MCF-7N cells (Appendix, Figure 3). Since this project was begun, evidence has accumulated that the sphingolipid ceramide as a key determinant in sensitivity to chemotherapeutic agents, with aberrant Cer signaling implicated in resistant to both chemo- and radiotherapy. In addition, Cer is able to inhibit PI3K-Akt activity through dephosphorylation of both Akt and downstream targets of Akt, and has been demonstrated to inhibit the activity of PKC-α placing Cer in the position to inhibit two key survival pathways in MCF-7 cells. Interestingly, we have previously demonstrated in MCF-7 cells that activation of PI3K can diminish the production of Cer and increase breast cancer cell survival in response to TNF-α. This led us to hypothesis that a lack of Cer signaling was contributing to chemoresistance in these cells. We used D-*erythro*-C8-ceramide to test whether treatment with exogenous Cer could restore the ability of the MCF-7TN-R cells to undergo cell death. Treatment of MCF-7 cell variants with Cer resulted in a virtually identical dose-dependent decrease in cell viability over 48 hr (Appendix, Fig. 4), with an IC_{50} of approximately 30 μM in both the TNF-sensitive and the TNF-resistant cell variants, demonstrating that Cer could reverse resistance in our cellular model. Cer was also able to decrease the long-term survival and colony-forming potential of both cell lines equally, as measured by an 8-day colony assay.

We also showed that the efficacy of Cer could be increased through with modification of the degree of unsaturation of the sphingoid backbone, and out of 5 novel Cer analogs tested (2*S*,3*R*)-(4*E*,6*E*)-2-octanoylamidooctadecadiene-1,3-diol was the most efficacious analog with an IC50 of 11.3μM. We determined that Cer-induced cell death was a result of induction of apoptosis both in our

chemosensitive and chemoresistant cell variants as measured by increased Annexin V staining, and that apoptosis occurred through activation of the mitochondria as seen by a release of cytochrome c into the cytosol and decreased mitochondrial membrane potential.

Since Cer has been shown to inhibit activity of both the PI3K/Akt and PKC-α, we investigated the effect of Cer treatment on these kinases. We found that treatment of MCF-7 cells with Cer decreased with phosphorylation of Akt in both a time-dependent and dose-dependent manner (Appendix, Fig. 5). Cer treatment did not appear to alter the phosphorylation (representative of activity) of PKC-α under the conditions tested (Appendix, Fig. 5), suggesting that one of the mechanisms by which Cer is able to convert our chemoresistant MCF-7 cell variant to a chemosensitive one is through inhibition of Akt survival signaling. The ability of Cer to inhibit MCF-7 cell viability may be related to Cer-mediated inhibition of Akt-stimulated NF-κB activity. Cer was also found to inhibit basal and TNF-α-stimulated NF-κB transcriptional activity as measured by a NF-κB reporter gene construct.

KEY ACCOMPLISHMENTS

- Demonstrated the importance of PI3K and PKC in the survival pathways of MCF-7 cell variants
- Demonstrated that Cer can induce apoptosis through activation of the mitochondria in our isogenic model of breast cancer resistance
- Demonstrated the importance of Cer signaling in MCF-7 cell chemosensitivity, possibly through inhibition of the PI3K/Akt/NF-κB survival pathway
- Development of "lead" ceramide analog shown to more effectively induce apoptosis and restore chemosensitivity to apoptosis-resistant MCF-7 cells

OUTCOMES

- Submission of manuscript (see Appendix for abstract) to Journal of Pharmacology and Experimental Therapeutics
- Submission of abstract for 2003-2004 annual meeting of American Association of Cancer Researchers
- Presentation of data at Tulane Health Science Center Research Days (April, 2003)
- Presentation of data at seminar Department of Pharmacology (April, 2003)
- Submission of patent application with Dr. Barbara Beckman and Dr. Robert Bittman (in progress)

Appendix Cover Sheet

Appendix

DAMD17-01-1-0432

Fig.1

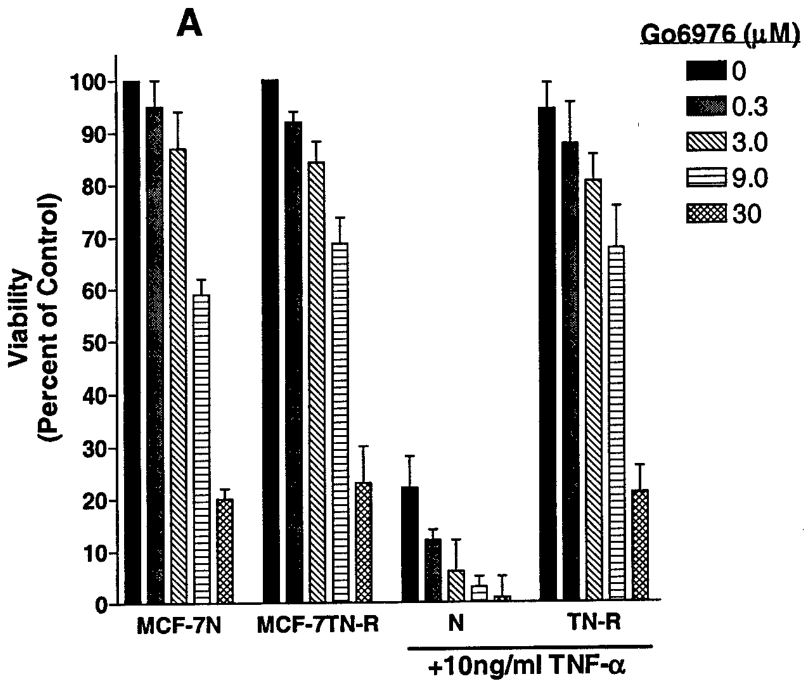
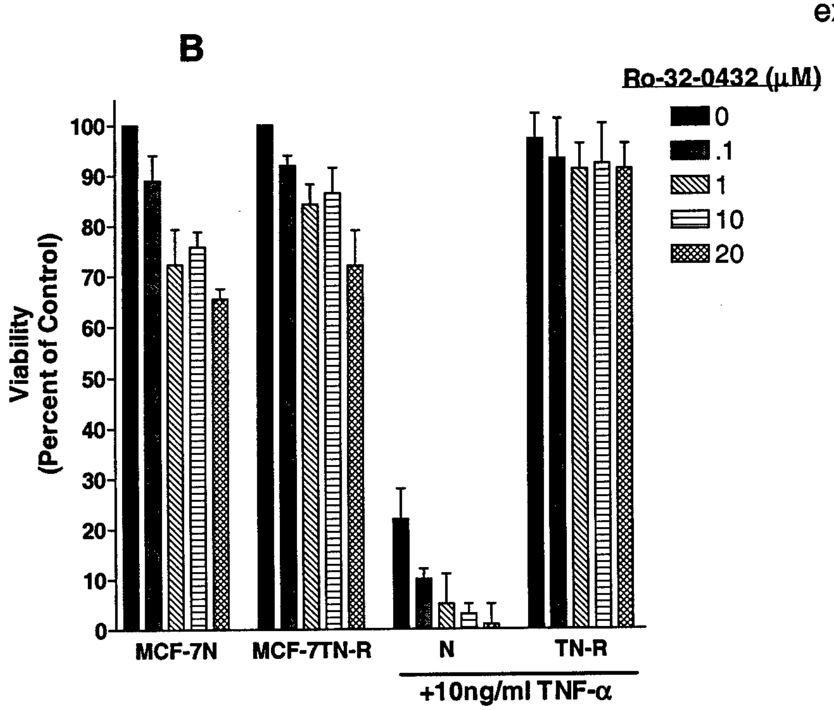


Fig.1 Effect of PKC inhibition in MCF-7 cells. MCF-7N or MCF-7TN-R variants were treatment with the indicated dose of PKC inhibitors (A) Go6976 or (B) Ro-32-0432 for 48 hr, with or without 10 ng/ml TNF-α. Cell viability was determined by MTT assay and is expressed as percent of control.



Appendix

Fig.2

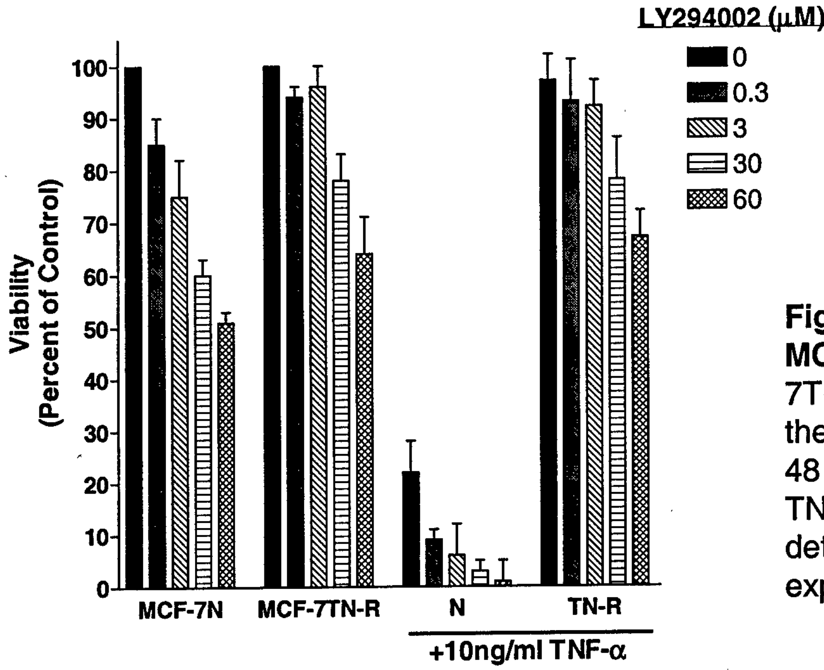


Fig.2 Effect of PI3K inhibition in MCF-7 cells. MCF-7N or MCF-7TN-R variants were treatment with the indicated dose of LY294002 for 48 hr, with or without 10 ng/ml TNF-α. Cell viability was determined by MTT assay and is expressed as percent of control.

Fig.3

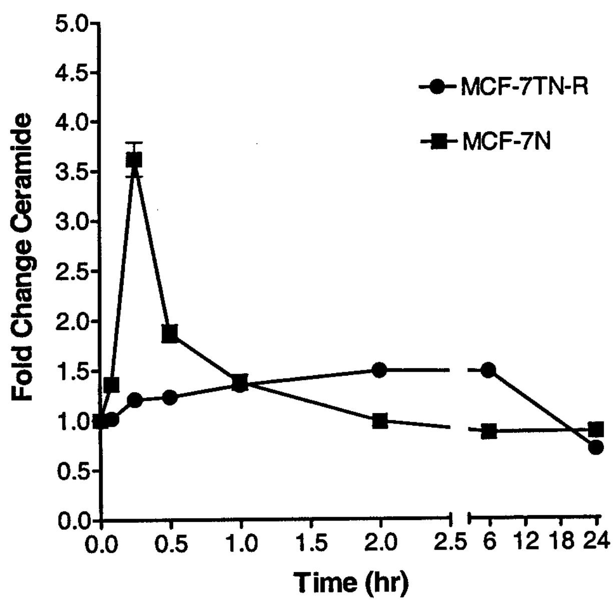


Fig.3 Resistant MCF-7 cells do not accumulate significant levels of ceramide following TNF-α treatment. MCF-7TN-R or MCF-7N cell variants were exposed to 10 ng/ml TNF-α for the indicated times. Cells were fixed in ice-cold methanol, harvested, and ceramide levels were measured. Changes in ceramide levels are expressed as fold change over vehicle-treated control (normalized to one).

Appendix

Fig.4

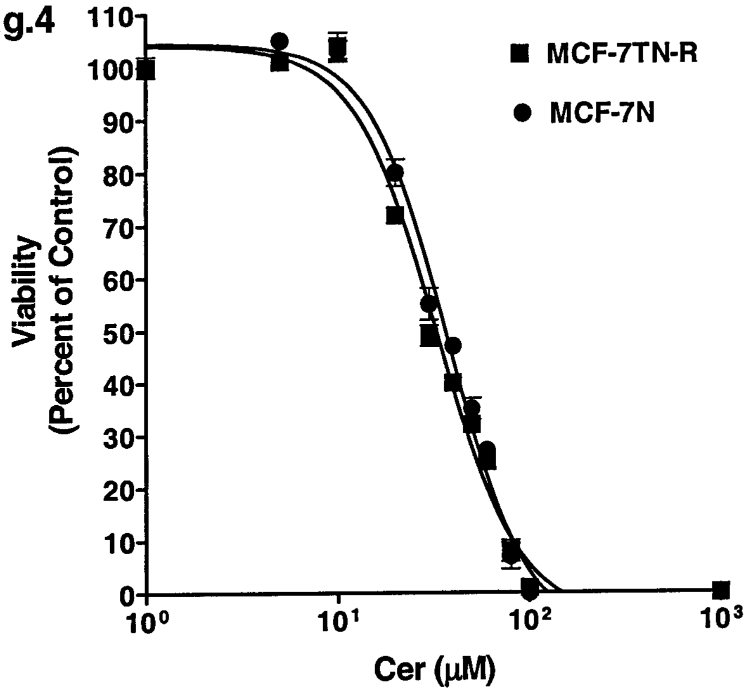


Fig.4 Cer treatment restores chemosensitivity in MCF-7TN-R cells. MCF-7 cells variants were incubated with the indicated doses of Cer for 48 hr. Following incubation, cell viability was estimated using the MTT assay. Data are presented as percent viability of vehicle-treated control cells.

Fig.5

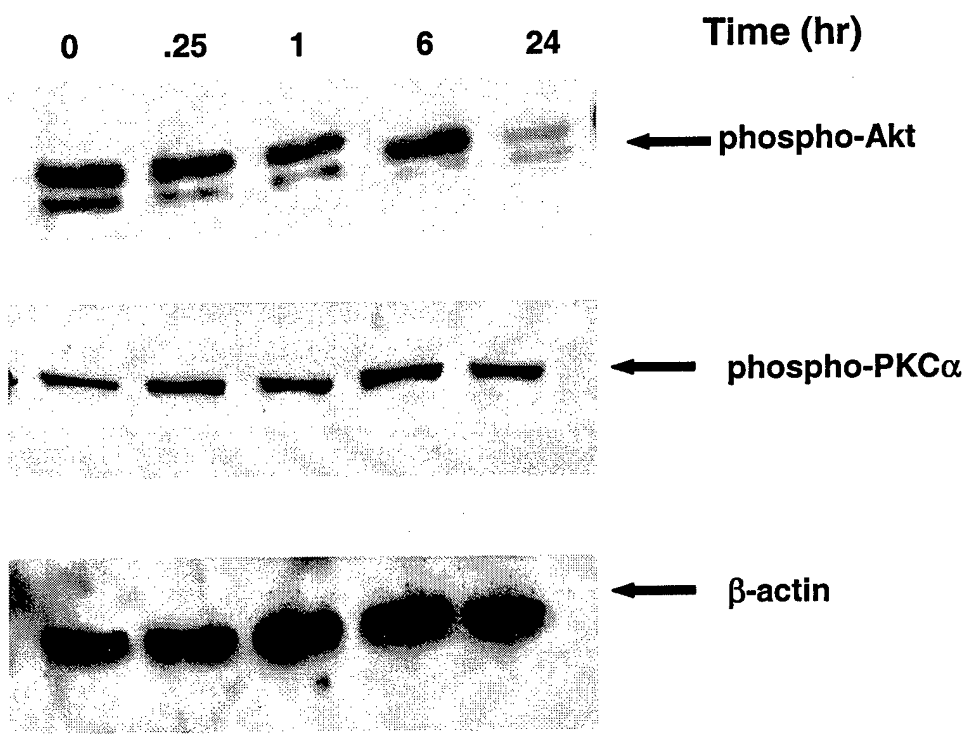


Fig.5 Ceramide dose-and time-dependently inhibits phosphorylation of Akt MCF-7TN-R variant was exposed to 30 μM ceramide for 24 hr. Cells were harvested, lysed, and proteins were detected using a standard immunoblotting protocol. Membranes were probes with either (A) anti-phospho-Akt, (B) anti-phospho-PKCα. or (C) anti-β-actin

Appendix

DAMD17-01-1-0432

Abstract of paper submitted to Journal of Pharmacology and Experimental Therapeutics

Recent evidence suggests a role for aberrant ceramide levels in the pathogenesis of cancer and chemoresistance, and indicates that manipulation of tumor ceramide levels may be a useful strategy in the fight against breast cancer. This study demonstrates that alterations in the degree and position of unsaturation of bonds in the sphingoid backbone of D-*erythro*-*N*-octanoylsphingosine (Cer) affect the antiproliferative ability of ceramide analogs in breast cancer cells. The most efficacious analog of Cer we tested is (2*S*,3*R*)-(4*E*,6*E*)-2-octanoylamidooctadecadiene-1,3-diol (4,6-diene-Cer), which contains an additional trans double bond at C(6)-C(7) of the sphingoid backbone. 4,6-Diene-Cer exhibited higher cytotoxicity than Cer in TNF-α-resistant (IC_{50} 11.3 μM vs 32.9 μM) and TNF-α-sensitive (IC_{50} 13.7 μM vs 37.7 μM) MCF-7 cells. 4,6-Diene-Cer was also more effective than Cer in inducing cell death in MDA-MD-231 and NCI/ADR-RES breast cancer cell lines (IC_{50} 3.7 μM vs 11.3 μM, and 24.1 μM vs 86.9 μM, respectively). 4,6-Diene-Cer caused a prolonged elevation of intracellular ceramide levels in MCF-7 cells, which may contribute to its enhanced cytotoxicity. Furthermore, treatment of MCF-7 cells with Cer or 4,6-diene-Cer resulted in induction of apoptosis by 8 hr via the mitochondrial pathway, as demonstrated by release of cytochrome c, loss of membrane asymmetry (measured by Annexin V staining), and a decrease in the mitochondrial membrane potential. Importantly, both Cer and 4,6-diene-Cer displayed selectivity toward transformed breast cells over non-transformed breast epithelial cells. These data suggest that these and other novel ceramide analogs represent potential therapeutic agents in breast cancer treatment.